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New Candidate Vaccines against Blood-Stage *Plasmodium falciparum* Malaria: Prime-Boost Immunization Regimens Incorporating Human and Simian Adenoviral Vectors and Poxviral Vectors Expressing an Optimized Antigen Based on Merozoite Surface Protein 1▽†

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Although merozoite surface protein 1 (MSP-1) is a leading candidate vaccine antigen for blood-stage malaria, its efficacy in clinical trials has been limited in part by antigenic polymorphism and potentially by the inability of protein-in-adjuvant vaccines to induce strong cellular immunity. Here we report the design of novel vectored *Plasmodium falciparum* vaccines capable of overcoming such limitations. We optimized an antigenic insert comprising the four conserved blocks of MSP-1 fused to tandemly arranged sequences that represent both allelic forms of the dimorphic 42-kDa C-terminal region. Inserts were expressed by adenoviral and poxviral vectors and employed in heterologous prime-boost regimens. Simian adenoviral vectors were used in an effort to circumvent preexisting immunity to human adenoviruses. In preclinical studies these vaccines induced potent cellular immune responses and high-titer antibodies directed against MSP-1. The antibodies induced were found to have growth-inhibitory activity against dimorphic allelic families of *P. falciparum*. These vectored vaccines should allow assessment in humans of the safety and efficacy of inducing strong cellular as well as cross-strain humoral immunity to *P. falciparum* MSP-1.

Attempts to generate protective blood-stage immunity to *Plasmodium falciparum* by vaccination in humans have met with limited success to date (18). The focus for most vaccine candidates has been on the induction of antibodies against merozoite antigens and merozoite surface protein 1 (MSP-1) in particular (24). Antibodies against the blood stage of *P. falciparum* are known to contribute to protective immunity in humans (40). However, the induction of antibodies to the 42-kDa portion of MSP-1 (MSP-1_{42}) appeared to be insufficient to provide protective immunity in humans in one study (39). Evidence from both animal models and humans (detailed below) suggests that cell-mediated immune responses to MSP-1 could be additionally required to induce protective immune responses.

During the process of merozoite invasion into erythrocytes, MSP-1 undergoes two proteolytic processing steps; following the first step, only MSP-1_{42} remains membrane bound, and a second cleavage of MSP-1_{42} into 33-kDa (MSP-1_{33}) and 19-kDa (MSP-1_{19}) portions is then required for erythrocyte invasion (4). MSP-1_{19} is a major target of protective antibodies, and MSP-1_{33} is a target of both $CD8^+$ T cells and $CD4^+$ helper T cells (11, 21, 25). Antibodies to MSP-1_{19} are thought to act though the direct inhibition of merozoite invasion into the red blood cell and via cytophilic antibody-mediated antibody-dependent cellular inhibition (24, 33). $CD4^+$ T cells specific to MSP-1_{33} are able to partially protect nude mice from lethal *Plasmodium chabaudi* and *Plasmodium yoelii* infections (53, 57), while transferred antibodies to MSP-1_{19} alone are unable to protect nude mice against *P. yoelii* (22). $CD4^+$ T cells against MSP-1_{33} play an important role in providing help for priming MSP-1_{19}-specific B cells in vaccine-induced protection against murine malaria (11), and depletion of $CD4^+$ T cells has been shown to reduce protection against *P. yoelii* (23).

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Following the discovery that MSP-1 is also expressed late in the liver stage (49), $CD8^+$ T cells directed against MSP-1_{33} have been shown to protect against *P. yoelii* in the preerythrocytic stage (11, 27). In addition, immune responses induced by immunization with nonlethal blood-stage parasites of *P. yoelii* have been shown to protect against sporozoite challenge, through $CD4^+$ and $CD8^+$ T cell mechanisms and at least partly through release of gamma interferon (IFN-γ) (2). This discovery that $CD8^+$ T cells mediate significant antiparasitic activity against the liver stage of *P. yoelii* provides an argument that similar mechanisms may occur in human *P. falciparum* malaria. Further suggestion of the role of cellular immunity in protection against *P. falciparum* comes from those studies in humans in which protective immunity has been associated with significant cellular immune responses to blood-stage parasites, in the absence of strong blood-stage antibody responses (42, 47). In the first study, the secretion of IFN-γ appeared to be associated with protection against blood-stage *P. falciparum* malaria (42), and in the second, the presence of polyfunctional T cells, secreting tumor necrosis factor alpha (TNF-α) and interleukin-2 (IL-2) in combination with IFN-γ when stimulated by blood-stage parasites, was shown to be associated with protection against *P. falciparum* (47). We therefore sought to develop a vaccine targeting MSP-1, which would induce strong cellular immune responses in conjunction with high antibody titers.

While inhibitory antibodies prevent MSP-1_{19} processing and erythrocyte invasion and appear to be beneficial to the human host, blocking antibodies act to inhibit the action of these beneficial antibodies (19). Enzyme-linked immunosorbent assays (ELISAs) and immunofluorescence assays (IFAs) are routinely used to quantify the responses to vaccination but give no functional information as to levels of invasion-inhibitory antibodies. Growth-inhibitory activity (GIA) assays measure the growth of *P. falciparum* in the presence of immune sera *in vitro*, and such assays may be of greater clinical relevance when assessing vaccines targeting regions of MSP-1. The definition of epitopes for inhibitory and blocking monoclonal antibodies has enabled the design of vaccines that aim to induce inhibitory, in preference to blocking, antibodies against MSP-1_{19} (13, 16). We investigated the inclusion of this approach in the design of MSP-1 antigens in this study.

Regions of MSP-1, such as MSP-1_{33}, show extensive polymorphism, and this divides those sequences into two sets of allelic families that demonstrate extensive diversification (55). Allelic variation has been shown to reduce antibody production by single-allele vaccine constructs to heterologous strains in humans (37) and to reduce protection against heterologous strain challenge in nonhuman primates (31). Moreover human T cell responses against some peptides representing these two major allelic types of MSP-1 have been found to be mutually inhibitory in *in vitro* assays, a phenomenon termed altered peptide ligand antagonism (28). Within the ~190-kDa protein sequence of MSP-1, blocks have been defined on the basis of their extensive, more limited, and minimal genetic diversity (55). For example in block 8 there is only 10% homology at the amino acid level, while in block 17, encoding MSP-1_{19}, there is 90% homology (55). Blocks 16 (MSP-1_{33}) and 17 (MSP-1_{19}) together encode MSP-1_{42}.

Virus-vectored vaccines are becoming established as relatively inexpensive, effective, and safe alternative vaccine platforms to conjugate protein-in-adjuvant vaccines (12, 20). We have previously demonstrated the induction of high-titer protective antibodies against *P. yoelii* rodent malaria by using a recombinant replication-incompetent form of human adenovirus serotype 5 (AdHu5) and modified vaccinia virus Ankara (MVA) as vaccine vectors in a heterologous prime-boost regimen (13). A heterologous human adenoviral regimen has recently been shown to induce a strong, polyfunctional, and protective T cell response against simian immunodeficiency virus (SIV) in rhesus macaques (30), and a homologous AdHu5 regimen expressing MSP-1_{42} has been shown to induce GIA in rabbits (5). Experience in the HIV vaccine field has emphasized the importance of avoiding preexisting antivector immunity when developing vectored vaccines (6). The immunogenicity of virus-vectored vaccines is reduced in the presence of preexisting vector-neutralizing antibodies against AdHu5 (6–8), but such antibodies do not reduce the immunogenicity of simian adenoviral vectors in humans (K. Ewer et al. unpublished data). We hypothesized that a heterologous chimpanzee adenoviral vector regimen may induce strong immune responses and be more suitable for clinical use. The simian adenoviruses C6, C7, and C9 have structural similarity and sequences close to those of human adenovirus 4 in subgroup E. These vaccine vectors have shown promise in preclinical vaccines against infections such as rabies and HIV (45, 59). An alternative simian adenovirus, AdCh63, which is closely related to C6, C7, and C9, has recently been found to be safe, immunogenic, and efficacious for human use when used to express the well-studied preerythrocytic vaccine candidate antigen ME-TRAP (Ewer et al., submitted for publication). We therefore assessed our MSP-1 vaccine constructs in simian adenovirus-vectored vaccines.

To develop an MSP-1 vaccine for clinical trials, we investigated the possibility of including in vectors (i) the conserved blocks of MSP-1, (ii) both allelic forms of MSP-1_{42}, and (iii) a modified MSP-1 sequence to improve antibody fine specificity and of using a variety of DNA-based virus-vectored vaccines, including several simian adenoviral serotypes. We show in animal models that both cellular and humoral immune responses can be generated by this approach. We propose that such responses may help to overcome some of the limitations of previous generations of vaccines against MSP-1.

MATERIALS AND METHODS

Antigen inserts. PfM115 and PfM128 were based on the structure of MSP-1. A schematic representation of the structures of the composite antigen inserts "PfM115" and "PfM128" is shown in Fig. 1. PfM128 is identical to PfM115 with the exception of the addition of the FVO block 17 (MSP-1_{19}). Full sequence details can be found in the supplemental material. The rationale for this novel antigen structure was an effort to maximize inclusion of conserved antigen sequences, include both alleles of MSP-1_{42}, and minimize polymorphic regions, glycosylation targets, and epitopes in block 17 that are thought to induce blocking antibodies.

Vaccines. The final antigen inserts were codon optimized for mammalian expression and synthesized by GeneArt GmbH (Regensburg, Germany). Viral vaccine vectors included modified vaccinia virus Ankara (MVA), human adenovirus serotype 5 (AdHu5), and the simian adenoviruses (SAd) serotypes 6 (C6), 7 (C7), 9 (C9), and 63 (AdCh63). PfM115 was cloned into SAd viral vectors C6, C7, and C9 using methods described previously (48). Insertion of PfM115 and PfM128 into AdHu5 and MVA and of PfM128 into AdCh63 was performed using methods previously described for other antigen inserts (13, 44). As the majority of experiments were performed using PfM115, all vaccines express PfM115 unless stated to express PfM128.

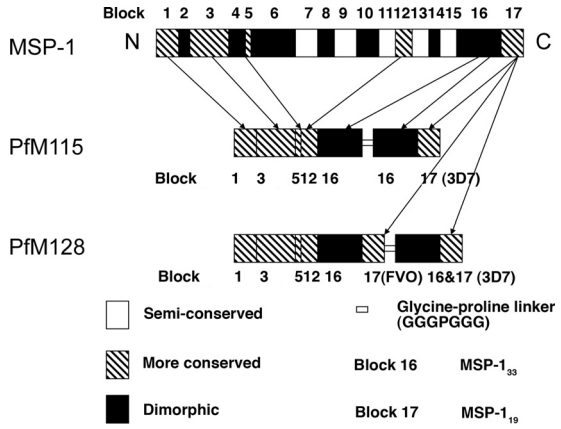

FIG. 1. A schematic representation of the design of the composite MSP-1 antigen inserts. The design of PfM115 and PfM128 was based on the structure of merozoite surface protein 1 and included the four more-conserved blocks (blocks 1, 3, 5, and 12) and FVO and 3D7 allelic variants of blocks 16 and 17 as shown.

Animals and immunizations. Groups of ≥6 female BALB/c and C57BL/6 mice (BMSU, Oxford University, United Kingdom) were 6 to 8 weeks of age at the start of the experiments. All procedures were performed in accordance with the terms of the United Kingdom Animals (Scientific Procedures) Act Project License and were approved by the University of Oxford Animal Care and Ethical Review Committee. Mice were immunized intradermally (i.d.) into both ears. Doses of vaccine used were 5×10^{10} viral particles (VP) of adenoviral vaccines and 5×10^7 PFU of MVA PfM115 unless otherwise stated. Doses of PfM128 vaccines were 10^{10} VP of adenoviral vaccines and 10^7 PFU of MVA PfM128 unless otherwise stated. Groups of ≥3 New Zealand White rabbits were immunized, and serum collection was performed by Agrobio (France). Doses of vaccine used were 5×10^{10} VP of adenoviral vaccines and 1×10^8 PFU (MVA PfM115) or 5×10^7 PFU (MVA PfM128) of orthopoxviral vaccines. All vaccines were administered i.d. unless otherwise stated. Viral vector vaccines were prepared in sterile, endotoxin-free phosphate-buffered saline (PBS). For comparison, two groups of six mice were immunized with a modified MSP-1_{19} protein (FVO allele) in PBS, complete Freund's adjuvant (CFA), or incomplete Freund's adjuvant. This was conjugated at the C terminus to the core domain of murine C4 binding protein (C4BP) (IMX108) (38) and was a kind gift from F. Hill (Imaxio, France). Three protein immunizations were administered subcutaneously (s.c.) at 2-week intervals. Three doses were administered. The first dose was formulated in PBS or CFA. Subsequent doses were formulated in PBS or incomplete Freund's adjuvant. All mouse data shown are representative of two or three experiments. Rabbit experiments were performed once only and data shown are from a single data set, with assays repeated to ensure technical validity.

In vitro GIA assay. A standardized *in vitro* growth inhibition activity (GIA) assay was performed at the GIA Reference Center (LMVR, NIH) as previously described (32). Rabbit IgGs from individual animals vaccinated with all PfM115 vaccines were tested against 3D7 and FVO parasites at 2.5 mg/ml and against 3D7 parasites at 10 mg/ml. Rabbit IgGs from animals vaccinated with AdCh63 PfM128, AdHu5 PfM128, and MVA PfM128 vaccines were tested against 3D7 parasites at 10 mg/ml.

ELISA. Recombinant glutathione *S*-transferase (GST) fusions of *P. falciparum* ETSR MSP-1_{19} and QKNG MSP-1_{19} were prepared as described previously (13). Protein was applied at an optimized concentration (2 μg/ml ETSR and 5 μg/ml QKNG) in PBS. Sera were diluted to 1:100 (preboost) or 1:1,000 (postboost), added in duplicate wells, and serially diluted. For monoclonal antibody (MAb) ELISAs, MAbs at a concentration of 5 μg/ml were applied in place of sera. The endpoint titers were taken as the *x* axis intercept of the dilution curve at an absorbance value three standard deviations greater than the optical density at 405 nm (OD_{405}) for naïve mouse or rabbit sera. Recombinant full-length His_6-tagged MSP-1 (a heterodimeric complex reconstituted from MSP-$1_{83/30}$ and MSP-$1_{38/42}$) and the 83-, 38-, and 42-kDa subunits of MSP-1 were prepared and purified as described elsewhere (15, 26). MSP-1_{42} ELISAs for correlation with GIA were performed on purified IgG using methods described elsewhere (35). Recombinant full-length MSP-1 was kindly provided by H. Bujard (ZMBH, Germany). All ELISAs were performed using previously published methods (13, 35, 58).

Immunofluorescence assay (IFA). Slides were prepared with a thin smear of *P. falciparum* schizonts from culture and fixed with 4% formaldehyde and 1% NP-40 for 15 min at room temperature. Rabbit sera were diluted 1:1,000 in PBS and incubated on slides for 45 min. Slides were washed with PBS and incubated with Alexa 488 goat anti-rabbit IgG for 30 min. Slides were then washed in PBS, DAPI (4′,6′-diamidino-2-phenylindole) was applied, and the slides were viewed under a fluorescence microscope.

Intracellular cytokine staining. Flow cytometry analysis of T cell phenotype was performed according to a protocol detailed in the supplemental material and based on methods published elsewhere (11). Data were analyzed using a Cyan ADP flow cytometer, FloJo (version 9), and SPICE (Mario Roederer). Background responses in unstimulated cells were subtracted from the stimulated responses prior to analysis.

Immunostaining. Chicken embryo fibroblasts were infected with MVA PfM115 or MVA PfM128 and incubated for 3 days. Infected cells were then fixed and permeabilized. Cells were incubated with inhibitory (12.8, 12.10, and 1E1), neutral (2F10), or blocking (111.4, 2.2) monoclonal antibody at 1:1,000 (5 μg/ml) for 1 h. Goat anti-mouse–horseradish peroxidase (HRP) (Amersham Biosciences) was applied and detected using chromogenic diaminobenzidine (DAB). Plaques were visible by eye and were imaged using a gel camera. Binding of antibody to PfM115 was determined by the presence (+) or absence (−) of visible stained MVA plaques.

Statistics. Statistical significance was analyzed using Prism version 5 (GraphPad Software Inc., CA) and STATA. Details of the statistical tests used for each analysis are listed in the supplemental material.

RESULTS

Antigen design: PfM115 and PfM128. A first-generation MSP-1 vaccine insert, termed PfM115, was designed to include the four relatively conserved blocks of MSP-1 (blocks 1, 3, 5, and 12) from the 3D7 strain of *P. falciparum.* These were encoded from the N to the C terminus, followed by block 16 from the Wellcome strain (FVO) and then blocks 16 and 17 from the 3D7 strain (Fig. 1). A flexible linker was encoded between the two forms of block 16. The FVO and 3D7 strains represent the alternative dimorphic families for block 16, which encodes MSP-1_{33}. Immunization with MSP-1_{33} has been shown to induce $CD8^+$ and $CD4^+$ T cells and provide essential T cell help for antibody responses to MSP-1_{19}, the target of protective antibodies in mice (11). Three amino acid substitutions were made in the latter fragment, as described elsewhere, to enhance inhibitory antibody induction and to attempt to reduce induction of blocking antibodies (16). A second-generation construct, termed PfM128, has previously been reported (13). PfM128 differs from PfM115 in the inclusion of the 19-kDa fragment (block 17) from the FVO strain of *P. falciparum* immediately C terminal to the FVO strain 33-kDa fragment in the insert (Fig. 1).

Cellular immune responses to vaccination. T cell responses have been associated with the control of blood-stage malaria in mice and humans (17, 42). IFN-γ secretion and T cell-mediated responses to blood-stage antigens are thought to be of importance in mediating protection against *P. falciparum* and *P. berghei* (42, 47, 60). We hypothesized that polyfunctional $CD8^+$ and $CD4^+$ T cell responses would be induced by immunization with heterologous prime-boost virus-vectored vaccines and that the use of simian adenoviral vectors in place of human adenoviral vectors would not compromise vaccine immunogenicity. We sought to characterize the T cell responses using polychromatic flow cytometry.

In order to establish whether simian adenovirus-vectored vaccines would induce T cell responses to MSP-1, mice were

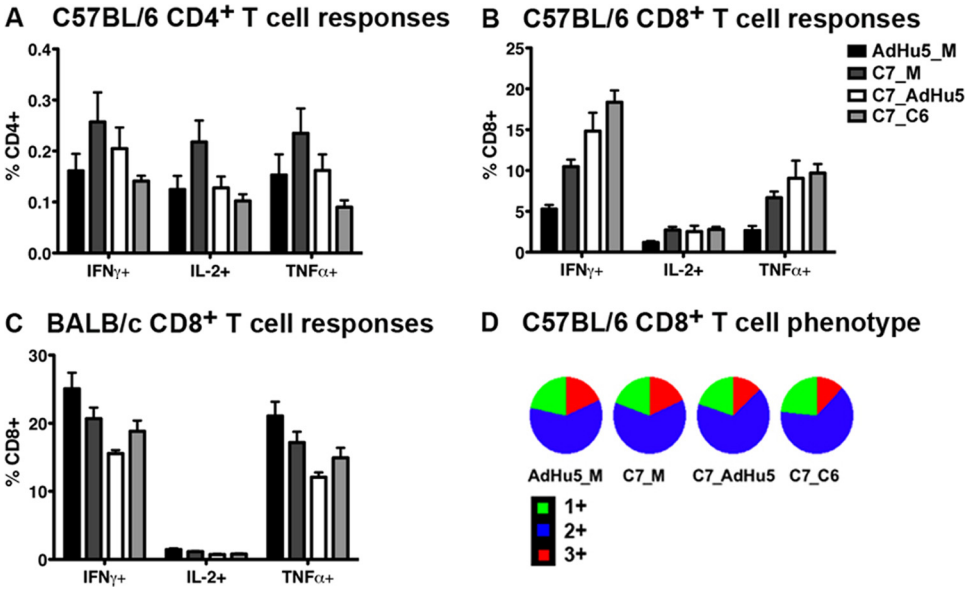

FIG. 2. T cell responses following immunization with viral vectors expressing PfM115. MSP-1-specific cytokine production from splenocytes of mice previously immunized with AdHu5 or C7 expressing PfM115, followed by a boost immunization at 8 weeks with C6, C7, or MVA (M) expressing PfM115 (prime_boost), was assessed at 2 weeks after the final immunization. Multiparameter flow cytometry was used to determine the total frequencies of IFN-γ-, TNF-α-, and IL-2-producing T cells. $CD4^+$ (A) and $CD8^+$ (B to D) T cell responses to vaccination are shown. The murine strains used are shown in the figure. In panel D The fraction of the total response comprising cells expressing one (+), two (++) or, three (+++) cytokines in the experiment for panel B is shown. Adenoviral vectors and poxviral vectors were given at doses of 5×10^{10} VP or 5×10^7 PFU, respectively. Data are means ± standard errors of the means (SEM) ($n = 6$).

immunized with a priming dose of adenovirus-vectored vaccine that expressed PfM115. A boost immunization of MVA (M), AdHu5, C6, or C7 expressing the PfM115 insert was administered 8 weeks later. Vector administration is denoted by prime_boost (e.g., AdHu5_M means AdHu5 PfM115 prime and MVA PfM115 boost). Where PfM128 was expressed as the antigen, this is additionally denoted (e.g., AdHu5_M PfM128). C9 has been demonstrated to cross-react with C7 neutralizing antibodies and was therefore not used as a boosting agent (48). Overlapping peptide pools were used to map T cell epitopes from PfM115 in mice (see Fig. S2 in the supplemental material). We identified a single immunodominant $CD8^+$ T cell epitope in the FVO MSP-1_{33} region of PfM115 in BALB/c mice and several weaker epitopes in C57BL/6 mice (see Table S1 in the supplemental material). The BALB/c epitope was consistent with H-2^d class I epitope prediction (http://www.syfpeithi.de) and with previous literature demonstrating T cell epitopes in the 33-kDa region of MSP-1 in other murine models (52). No $CD4^+$ T cell epitope was identified in BALB/c mice. A single $CD4^+$ T cell epitope was found in C57BL/6 mice (see Table S1 in the supplemental material).

All prime-boost regimens expressing PfM115 were found to induce T cell responses in mice (Fig. 2). $CD4^+$ T cell responses were measured in C57BL/6 mice, and no significant differences in $CD4^+$ IFN-γ^+, TNF-α^+, or IL-2^+ T cell responses were found between groups receiving PfM115 in different vaccine vectors (Fig. 2A). Heterologous adenoviral regimens showed a trend toward increased $CD8^+$ TNF-α^+ and IFN-γ^+ T cell responses, while a C7 simian adenoviral prime induced stronger $CD8^+$ IL-2^+ T cell responses in C57BL/6 mice ($P < 0.05$) (Fig. 2B). However, though these differences were replicated on repetition of the experiment in C57BL/6 mice, when the vaccines were administered to BALB/c mice, this effect was not seen (Fig. 2C). Overall, the replacement of a human adenoviral vector with a simian adenoviral vector did not appear to alter cellular immune responses. A proportion of T cells were polyfunctional and capable of coexpressing the cytokines TNF-α, IFN-γ, and IL-2 (Fig. 2D). The majority of these T cells were capable of producing both IFN-γ and TNF-α (represented as blue in pie charts), with a smaller number of polyfunctional triple-positive, IL-2-producing cells (shown in red).

Mice were immunized with vaccines expressing the second-generation antigen PfM128. AdHu5 and AdCh63 viral vectors expressing PfM128 were administered to mice and boosted 8 weeks later by immunization with MVA (M) expressing PfM128. $CD8^+$ and $CD4^+$ IFN-γ^+, TNF-α^+, and IL-2^+ responses were not compromised by the replacement of AdHu5 with AdCh63 PfM128 (Fig. 3). $CD8^+$ cytokine responses tended to increase with AdCh63_M PfM128 compared with AdHu5_M PfM128; these differences were significant only in the case of IL-2 ($P < 0.05$, Mann-Whitney test) (Fig. 3B). Polyfunctional analysis demonstrated a small but significant increase in both polyfunctional $CD4^+$ and $CD8^+$ T cells with the AdCh63 prime ($P \leq 0.01$, Mann-Whitney test) (Fig. 3C and D). However, once again neither the qualitative nor the quantitative improvement in $CD8^+$ T cell responses with AdCh63 was replicated in BALB/c mice (data not shown), suggesting that overall responses are not altered by replacement of AdHu5 with AdCh63.

Humoral responses against MSP-1_{19} following a single immunization. In order to establish the level of the antibody response to MSP-1 induced by simian adenovirus-vectored vac-

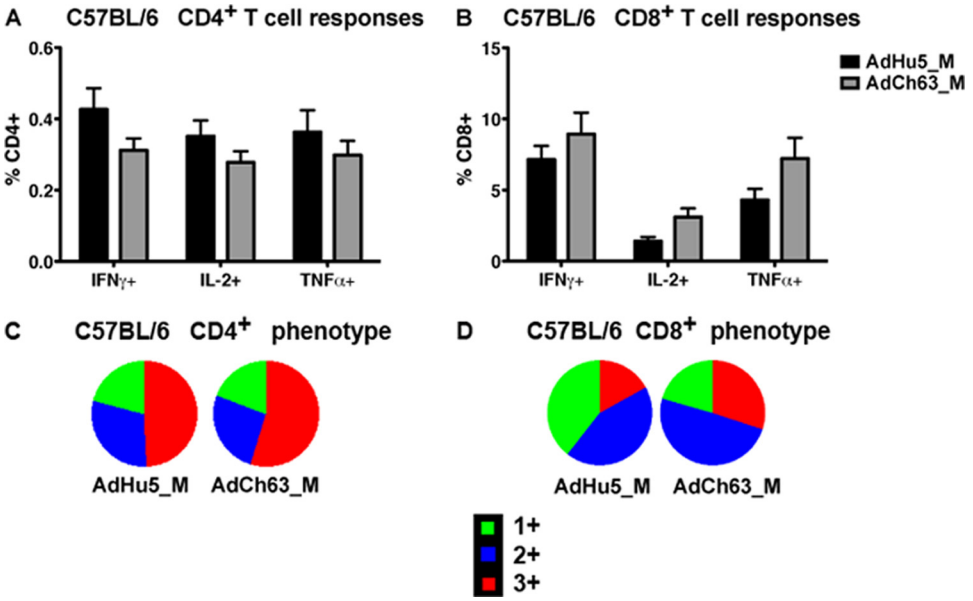

FIG. 3. T cell responses following immunization with viral vectors expressing PfM128. MSP-1-specific cytokine production from splenocytes of C57BL/6 mice previously immunized with AdHu5 or AdCh63 expressing PfM128, followed by a boost immunization at 8 weeks with MVA (M) expressing PfM128 (prime_boost), was assessed at 2 weeks after the final immunization. Multiparameter flow cytometry was used to determine the total frequencies of IFN-γ-, TNF-α-, and IL-2-producing T cells. $CD4^+$ (A and C) and $CD8^+$ (B and D) T cell responses to vaccination are shown. Panels C and D show the fraction of the total response comprising cells expressing one (+), two (++), or three (+++) cytokines. Adenoviral vectors and poxviral vectors were given at doses of 10^{10} VP or 10^7 PFU, respectively. Data are means ± SEM ($n = 6$).

cines, BALB/c mice were immunized with a variety of vaccine regimens. The BALB/c strain of mouse was chosen because antibody responses were higher than those seen in C57BL/6 mice, despite the absence of a detectable $CD4^+$ T cell epitope in this strain. This appears to be a feature of the antibody responses of BALB/c mice in general and was not particular to this antigen (13). It appears that $CD4^+$ T cell responses were induced in BALB/c mice, as antibody class switching occurred as evidenced by the presence of IgG, but these responses were below the detection limit of our cellular assays. The immunogenicity of AdHu5 was compared with those of the simian adenoviral vectors, C6, C7 C9, and AdCh63. MSP-1_{19} is known to be the target of IgG-mediated immunity in mice and humans (1, 11), and ELISAs were therefore performed against this region of MSP-1 unless otherwise stated. Antibody titers were monitored following a single immunization and were found to increase over time, as seen previously with *P. yoelii* MSP-1_{42} vaccines (13). The replacement of a single dose of AdHu5 with the simian vector C6, C7, or C9 did not lead to a significant difference in the total IgG antibody titers to MSP-1_{19} that were achieved following immunization, though there was a trend to a weaker antibody response when immunization was with C9 (Fig. 4A). Similarly, when mice were immunized with AdHu5 PfM128 or AdCh63 PfM128, no difference was seen between groups (Fig. 4B). This result differs from previous work with the *P. berghei* circumsporozoite malaria antigen in which significantly weaker antibody responses were found when C6 was used as a priming vector (46). The trend toward a weaker response with C9 (also known as C68) has been documented previously with rabies vaccines administered subcutaneously (59). These data demonstrate that total IgG immune responses to *P. falciparum* MSP-1_{19} are mostly preserved when AdHu5 is replaced with simian adenoviral vaccine vectors.

Humoral responses against MSP-1_{19} following heterologous prime-boost immunization. We have previously shown that a heterologous poxviral boost can improve antibody responses to *P. yoelii* MSP-1_{42} (13). We also reported that adenoviral vectors prime stronger antibody responses than the orthopoxviral vector MVA (13). We hypothesized that a heterologous adenoviral boost might induce a stronger antibody response than a poxviral boost. A boost immunization was administered, and antibody responses to MSP-1_{19} for all groups combined were compared to preboost titers. We found a significant increase in the antibody titers following a heterologous boost immunization (Fig. 4C); however, the replacement of MVA with a heterologous adenoviral vector did not increase total IgG responses. In order to confirm that the absence of differences between the vaccine regimens was not due to the relatively high vaccine doses, this experiment was repeated using a lower-dose regimen in order to induce suboptimal titers. There were still no significant differences between antibody responses to different viral vector regimens (see Fig. S3 in the supplemental material). At the peak of the response, which was at week 10, all mouse IgG isotypes were detected with all vaccine regimens (Fig. 4D). Heterologous simian adenoviral vectors and MVA are thus equally effectively at boosting antibody responses to MSP-1_{19} in a prime-boost vaccine regimen. As proof of principal, one regimen (AdHu5_M PfM115) was compared to three doses of MSP-1_{19} protein conjugated to murine C4BP core domain (IMX108) (13, 38). AdHu5_PfM115 was found to be as immunogenic as protein in Freund's adjuvant and significantly more immunogenic than protein in PBS alone (see Fig. S1 in the supplemental material).

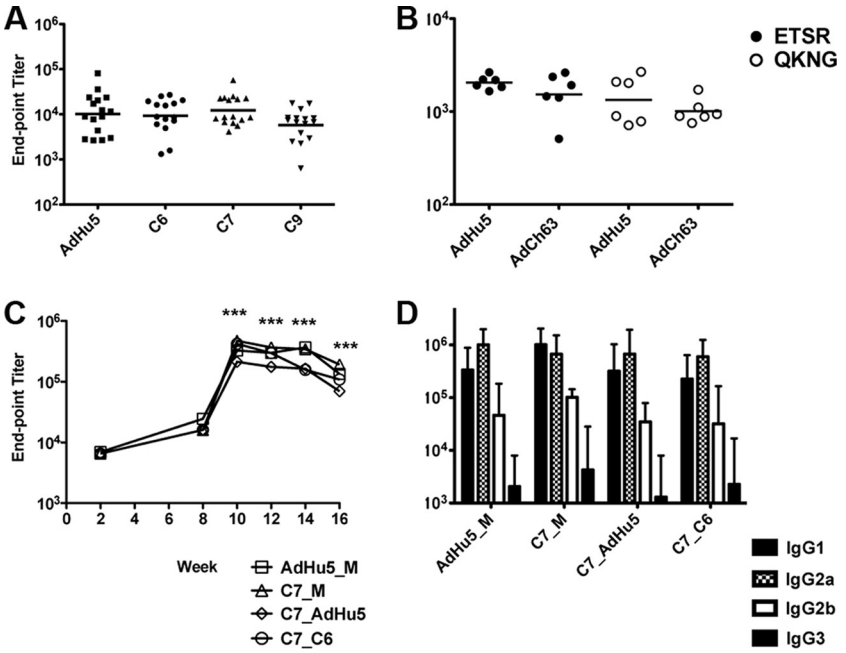

FIG. 4. Vaccine-induced antibody responses to MSP-1_{19}. BALB/c mice were immunized with adenoviral vectors (AdHu5, C6, C7, and C9), and total IgG titers to GST-MSP-1_{19} (ETSR) were measured by ELISA (unless stated otherwise). (A) Comparison of antibody responses at week 8 following a single immunization with 5×10^{10} adenoviral particles (VP) expressing PfM115. (B) Comparison of antibody responses at week 2 following a single immunization with 10^{10} VP expressing PfM128. (C) Total IgG responses (geometric mean titer [GMT]) over time following immunization as for panel A, followed at week 8 by a boost with 5×10^7 PFU MVA PfM115 (M) or 5×10^{10} VP AdHu5 or C6 PfM115 as shown (prime_boost). (D) Isotype ELISAs were performed at week 10 on samples from panel C. Total IgG titers to GST–MSP-1_{19} ETSR (A to D) and GST-MSP-1_{19} QKNG (C) were measured. Geometric mean titers (± 95% confidence intervals [CIs]) are shown. ***, Different from week 8 GMT ($P < 0.001$).

Humoral responses against MSP-1 in rabbits. In order to obtain sufficient sera for assays of parasite growth inhibition, the prime-boost immunization regimens described above were administered to New Zealand White rabbits. Strong antibody responses to MSP-1_{19} were found in all groups following a heterologous adenoviral or orthopoxviral boost (see Fig. S4A in the supplemental material). Antibody responses were increased significantly by the boost immunization. No significant differences were found between immunization regimens at any individual time point or overall. Antibodies were analyzed at week 10 and were detected to all regions of MSP-1 contained in the vaccine insert, with no significant differences between regimens (see Fig. S4C in the supplemental material). MSP-1_{83} incorporates blocks 1, 3, and 5 while MSP-1_{38} incorporates block 12 and MSP-1_{42} incorporates blocks 16 and 17. The proteins covering these three regions were based on the 3D7 allelic form. Antibody responses to MSP-1_{30} were not assessed, as this block was not included in the vaccine. Responses to the entire full-length MSP-1 molecule (see Fig. S4B in the supplemental material) did also not differ significantly between the vaccine groups.

Cross-strain humoral immunogenicity against MSP-1_{19}. Four amino acids have been found to differ between the different allelic variants of MSP-1_{19} and are thought to be important in antigen processing (34). These changes are E → Q at amino acid (aa) 1644 and TSR → KNG at aa 1691, 1700, and 1701. The allelic variants are therefore conventionally referred to as "ETSR" (3D7/Mad20 strain) or "QKNG" (FVO/Wellcome/K1/FCR3 strain). We first investigated whether antibodies produced in response to PfM115 immunization were specific to the allelic variant of the vaccine (3D7 [ETSR]) or whether they also had activity against the heterologous allelic variant (FVO [QKNG]). ELISAs were performed on sera collected at week 10 from mice immunized with all prime-boost PfM115 combinations in order to determine the total IgG titers against MSP-1_{19} ETSR and MSP-1_{19} QKNG. Antibody responses to the alternative allelic variant of MSP-1_{19} (QKNG) correlated with the homologous allelic antibody response (ETSR) in mice, and a stronger correlation was seen in rabbits (Fig. 5A and B). Such differences in cross-reactive antibody responses, dependent on the species immunized, have been reported previously for protein vaccines based on one or two alleles on the apical membrane antigen 1 (AMA-1) vaccine candidate (36). We hypothesized that the QKNG responses could be further improved with PfM128, which includes both allelic variants of MSP-1_{19}. Following immunization with AdHu5 PfM128 and MVA PfM128, total IgG responses to QKNG MSP-1_{19} in mice were significantly increased, while responses to ETSR MSP-1_{19} were maintained (Fig. 6). Mice immunized with this regimen showed a stronger correlation between ETSR and QKNG MSP-1_{19} ELISA titers than those immunized with PfM115 (Fig. 5A and C). A strong correlation in rabbits was maintained, similar to that seen with PfM115 (Fig. 5B and D).

Humoral responses to *P. falciparum*. Immunofluorescence assays (IFAs) were performed in order to determine if these

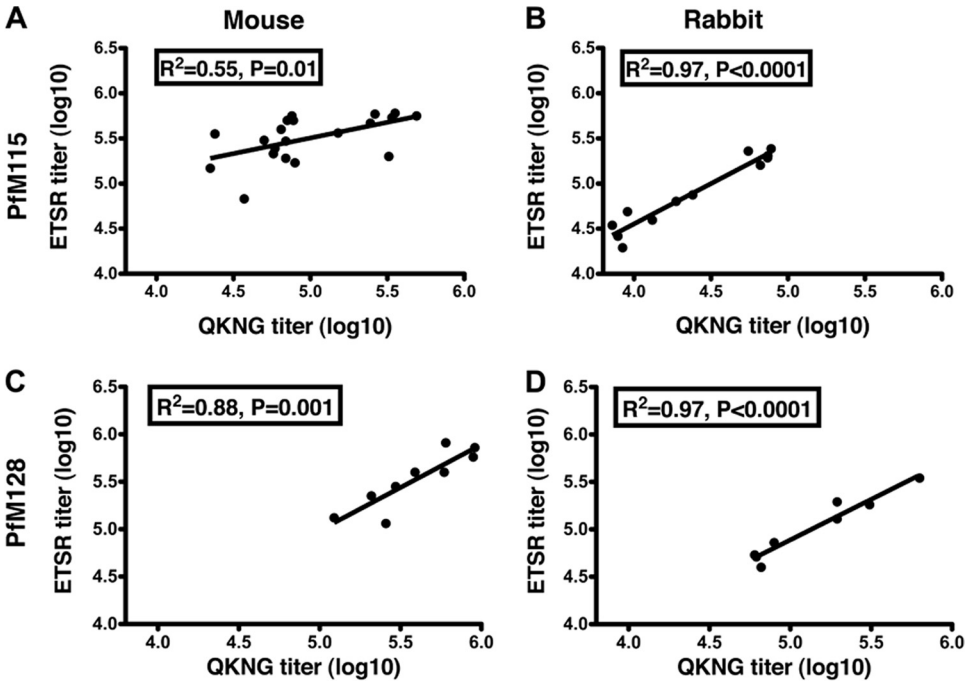

FIG. 5. Antibody responses to MSP-1 allelic variants. BALB/c mice (A and C) and New Zealand White rabbits (B and D) were immunized with vaccines incorporating the 3D7 allelic variant in PfM115 (A and B) or both allelic variants in PfM128 (C and D) of MSP-1_{19}. Mice were immunized as described for Fig. 4 and 6. Rabbits were immunized as described for Fig. 7. Animals received a range of prime_boost immunization regimens expressing PfM115 or AdHu5 PfM128 and a boost immunization of MVA PfM128 (C and D). Sera were taken at week 10. Total IgG ELISAs were performed for GST–MSP-1_{19} ETSR (3D7 allelic variant) and GST–MSP-1_{19} QKNG (FVO allelic variant). Antibody titers from individual animals and linear regression lines are shown, along with Pearson rank correlations (r^2) and *P* values.

antibodies were able to bind MSP-1 in its native form in *P. falciparum*. Sera were taken preimmunization and at 2 weeks after the boost immunization from all those rabbits immunized with vaccines expressing PfM115, and IgG was purified. Sera from immunized rabbits from all groups bound *P. falciparum* at a dilution factor of 1:1,000 (Fig. 7A), while preimmunization rabbit IgG did not. Sera were also taken at 2 weeks after the boost immunization from all those rabbits immunized with AdHu5_M PfM128, and a further IFA was performed. Sera were tested directly on slides from 3D7 and FCR3 parasites, and the IFA titer was found to be 1:6,400 against both parasite strains (median; see Fig. S5 in the supplemental material).

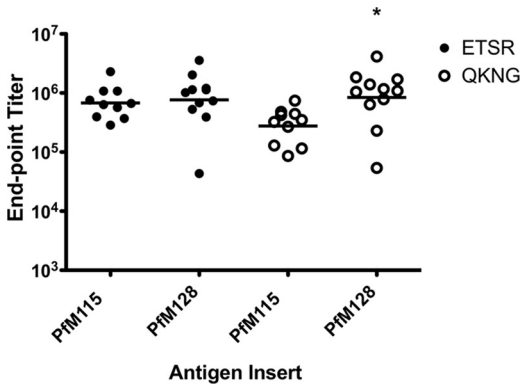

FIG. 6. Vaccine-induced antibody responses to MSP-1_{19} following AdHu5_M PfM115 or AdHu5_M PfM128. BALB/c mice were immunized with 10^{10} adenoviral particles of AdHu5 expressing either PfM115 or PfM128 as indicated and were boosted at week 8 by immunization with 10^7 PFU of MVA expressing the same antigen. Sera were taken at 2 weeks following the final immunization, and total IgG titers to GST–MSP-1_{19} (ETSR or QKNG, as shown) were measured by ELISA. *, different from PfM115 QKNG ($P < 0.05$).

The activity of antibodies against *P. falciparum in vitro* was established using a standard growth inhibition activity (GIA) assay (32). AdHu5_M PfM128 immunization of BALB/c mice has previously been shown to induce GIAs of 70% against 3D7 strain and 85% against FVO strain *P. falciparum* (13). BALB/c mice were immunized with AdHu5_M PfM115 as previously, and the GIAs were 46% against 3D7 parasites and 52% against heterologous FVO parasites. In rabbits, PfM115 vaccine regimens demonstrated efficacy against the *in vitro* growth of *P. falciparum* (Fig. 7C and D). Antibody titers to recombinant 3D7 MSP-1_{42} were found to have a sigmoidal relationship with the 3D7 GIA of purified IgG from rabbits (Fig. 7B). AdHu5_M PfM115 was administered intramuscularly (i.m.) (group A§) and i.d. (group B), and there was no statistical difference by the Mann-Whitney U test, but there was less variation in GIA at 10 mg/ml with i.m. administration (coefficient of variation = 6%) than with intradermal administration (coefficient of variation = 50%) (Fig. 7C). Vectors were therefore administered i.m. to subsequent groups of rabbits (group C§, AdHu5_M PfM128; group D§, AdCh63_M PfM128). At 10 mg/ml no significant differences in GIA were found between different viral vector combinations, or between those expressing PfM115 or PfM128 by the Mann-Whitney U test or one-way analysis of variance (ANOVA) (Fig. 7C). GIA was also

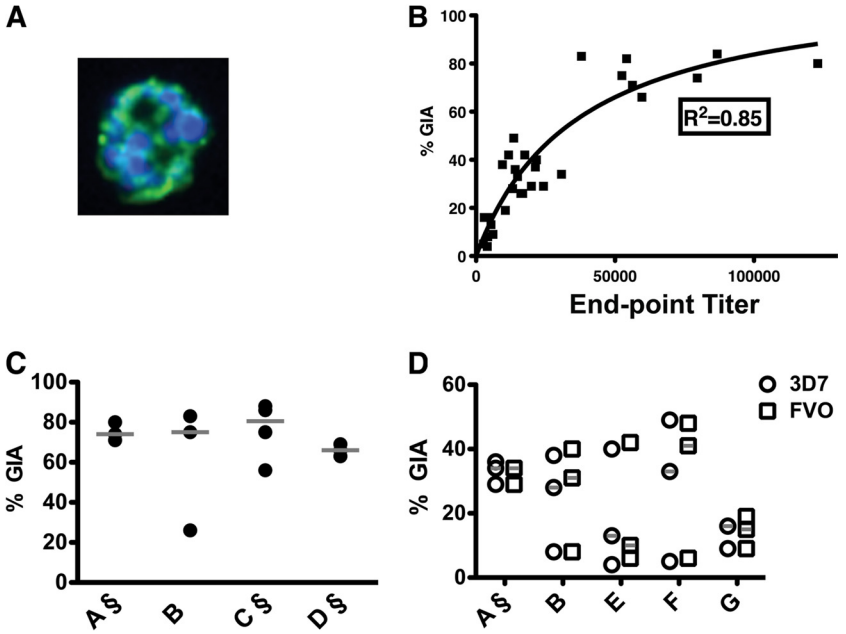

FIG. 7. Functional antibodies against whole *P. falciparum.* Rabbits (n = 3/group) were immunized with AdHu5 or C7 expressing PfM115 and boosted 8 weeks later with MVA PfM115 (M), AdHu5, or C6. Alternatively, rabbits were immunized with AdHu5 or AdCh63 expressing PfM128 and boosted with MVA expressing PfM128. Doses used were 5×10^{10} VP (adenoviruses) or 10^8 PFU (MVA). Both vaccines were administered i.d. or i.m. (§). Groups were as follows: A§, AdHu5_M PfM115 i.m.; B, AdHu5_M PfM115 i.d.; C§, AdHu5_M PfM128; D§, AdCh63_M PfM128; E, C7_M PfM115 i.d.; F, C7_C6 PfM115 i.d.; G, C7_AdHu5 PfM115 i.d. Purified polyclonal IgG for GIA was obtained from sera collected at 2 weeks after the final immunization. (A) IFAs were performed with sera from immunized rabbits. Areas in green are stained with anti-rabbit IgG secondary antibody, while areas in blue are stained with DAPI. No significant green staining was seen with naïve sera. A representative slide from a rabbit from group A is shown (1:1,000). (B) GIA responses were correlated with total IgG ELISA titer to 3D7 MSP-1_{42}. (C) GIA against 3D7 strains *in vitro* at 10 mg/ml IgG was determined. (D) GIA against 3D7 (circles) and FVO (squares) at 2.5 mg/ml IgG was determined. Individual and median values are shown.

tested at 2.5 mg/ml using sera from rabbits vaccinated with PfM115 regimens (group A, AdHu5_M PfM115 i.m.; group B, AdHu5_M PfM115 i.d.; group E, C7_M PfM115 i.d.; group F, C7_C6 PfM115 i.d.; group G, C7_AdHu5 PfM115 i.d.). There was no significant difference in GIA by the Mann-Whitney U test when homologous or heterologous parasite strains were used in this assay, and there was no significant difference between groups receiving different regimens when assessed by one-way ANOVA (Fig. 7D [circles represent 3D7 parasites and squares FVO]). Although GIA has been reported to be induced by antibodies targeting multiple regions of MSP-1 (58) and despite the induction of antibodies against MSP-1_{83} and MSP-1_{38} (see Fig. S4C in the supplemental material), the functional antibody responses appeared to be directed to the MSP-1_{19} region of the vaccine, as demonstrated by the complete reversal of GIA when recombinant MSP-1_{19} protein was applied to wells containing a mixture of parasites and immunized rabbit sera (see Fig. S6 in the supplemental material).

Aiming to minimize the induction of blocking antibodies. It has previously been suggested that a successful vaccine might be one designed in such a way as to optimize inhibitory antibodies while at the same time minimizing blocking antibodies by using specific amino acid substitutions (19). These have been previously incorporated into a protein-in-adjuvant vaccine and been found to improve immune responses and GIA (16). In PfM115 and PfM128, two cysteine residues in the sequence of MSP-1_{19} were replaced (in both alleles in PfM128) in order to disrupt a disulfide bond and attempt to minimize the induction of blocking antibodies and enhance antigen processing. These were the C12I and C28W changes (16). A further amino acid change, S3A, was also included in order to remove a potential N glycosylation site. N glycosylation of *P. falciparum* blood-stage proteins is rare, and the removal of glycosylation sites has been shown to improve antibody titers, antibody avidity, and protective efficacy of a recombinant MSP-1_{42} vaccine in nonhuman primates (54). We applied a panel of murine monoclonal antibodies specific for epitopes within the MSP-1_{19} domain to the poxviral vaccine viruses (MVA PfM115 and MVA PfM128) in a plaque immunoassay. We found that the MSP-1 protein expressed by MVA PfM115 and MVA PfM128 bound the inhibitory antibodies 12.10 and 12.8, the neutral antibody 2F10, and the blocking antibodies 7.5 and 1E1. The PfM115 antigen expressed by MVA failed to bind the blocking antibody 111.4. Proteins expressed by both MVA PfM115 and MVA PfM128 failed to bind the blocking antibody 2.2 (see Table S2 in the supplemental material). The binding to 111.4 by MSP-1 antigen expressed by MVA PfM128 but not MVA PfM115 was as expected because this antibody binds to an epitope present only in the QKNG allele (but not ETSR) of MSP-1_{19} which is not present in PfM115 but is present in PfM128 (9). Overall, the abolition of binding to MAb 2.2 showed that this combination of amino acid substitutions can in part remove binding to some, but not all,

known blocking antibody epitopes while maintaining inhibitory epitopes.

DISCUSSION

We have shown here the induction of T cell- and antibody-mediated immunogenicity against *P. falciparum* MSP-1 with viral vector vaccines. In the field of vaccine research it is challenging and probably unreliable to compare directly data generated in different laboratories, but in a direct comparison in our laboratory we found our initial regimen (AdHu5_M PfM115) to be equivalent to a protein vaccine administered in Freund's adjuvant—a formulation which, although highly immunogenic, is unsuitable for human use. The field of virus-vectored vaccines is now providing a platform for inducing strong cellular and humoral immune responses without the need for potentially reactogenic chemical adjuvants. To generate vectored vaccine candidates suitable for clinical assessment in the challenging field of blood-stage malaria vaccines, we have assessed the suitability of four new vaccine design features for MSP-1. These are a detailed assessment of simian adenoviral vectors as alternatives to the widely used AdHu5 serotype; the use of the four N-terminal conserved regions to try to generate T cell responses to more conserved rather than very variable regions of MSP-1; the inclusion of two allelic variants of the C-terminal MSP-1_{42} arrayed in tandem in a vectored insert; and the introduction of recently described point mutations in the 19-kDa fragment(s), a major target of protective antibodies, to enhance overall immunogenicity and reduce the likelihood of developing unwanted blocking antibodies.

We have demonstrated here that replacement of AdHu5 with a simian adenoviral vector compromises neither antibody nor T cell responses in animal models. We have also shown that MSP-1-specific $CD4^+$ T cells can be induced in C57BL/6 mice by all vaccination regimens tested. The proportion of $CD4^+$ T cells that are polyfunctional and express the cytokines IFN-γ, TNF-α, and IL-2 has been found to be a marker of the protective efficacy of the $CD4^+$ T cell response in murine leishmaniasis and human malaria (10, 47), and vaccine-induced polyfunctional T cells appear to be more durable than less-polyfunctional T cells in murine models of preerythrocytic malaria (44). We found little difference in the magnitude of the response, defined by these cytokines, for $CD4^+$ T cells induced by diverse vector-based heterologous prime-boost immunization regimens, but in some cases (e.g., AdCh63_M PfM128) a more polyfunctional profile of $CD4^+$ T cells was observed with simian than with human adenoviral vectors.

Studies in the *P. yoelii* model have demonstrated the importance of immunity to MSP-1, not only at the blood stage but also in preerythrocytic stages of parasite development (27). In agreement with other murine data (2, 27), we recently demonstrated the importance of $CD8^+$ T cell responses against *P. yoelii* MSP-1_{33} and their ability to partially control parasite growth at the liver stage of infection, indicating that the induction of such T cell responses by clinically relevant vaccine vectors may be of protective importance (11). The importance of polyfunctional $CD8^+$ T cells has been suggested in the field of HIV research, where the heterologous adenovirus-vectored vaccine combination of recombinant AdHu26 and AdHu5 was found to induce stronger, more polyfunctional $CD8^+$ T cell responses than a homologous AdHu5 regimen. These responses were associated with increased protection against an SIV challenge in rhesus macaques (30). In addition, recent work has shown evidence of GIA and T cell responses using a homologous AdHu5 vaccine expressing MSP-1_{42} (5). However, the continued clinical development of AdHu5 may be problematic, and a heterologous simian adenoviral (SAd_SAd) or simian adenoviral and MVA (SAd_M) regimen could therefore have an advantage. T cell responses have rarely been reported in preclinical studies of MSP-1 protein-in-adjuvant vaccines but have been shown following AdHu5 vector immunization (5). Here we show that induction of polyfunctional $CD8^+$ T cell responses to *P. falciparum* MSP-1 is not compromised by replacement of AdHu5 with a simian adenoviral vector, and some responses even appeared to be enhanced in C57BL/6 mice. Antibody titers and inhibition of parasite growth were also maintained with simian adenovirus-vectored vaccines. The median magnitude of GIA against 3D7 parasites using 10 mg/ml IgG was 74% (interquartile range [IQR], 64.5 to 81.5%) across all four groups tested. Other studies have previously shown induction of comparable levels of GIA using sera from rabbits immunized with MSP-1_{42} protein in Freund's adjuvant (complete and incomplete) (3) and following viral vector immunization (5).

The concentration of T cell epitopes in the MSP-1_{33} region is consistent with published data suggesting that T cell epitopes are often found in this region in *P. falciparum* in humans and *P. yoelii* in mice (13, 25, 52). It was on the basis of such published data that we originally decided to include both alleles of MSP-1_{33} in the composite antigen constructs. These vaccines differ from previous MSP-1 protein-based vaccines, usually based on MSP-1_{19} or MSP-1_{42}, by the inclusion of the four relatively conserved blocks 1, 3, 5, and 12. These were included in an attempt to induce conserved T cell responses that would transcend allelic differences. Despite the induction of antibodies to the four conserved blocks, we observed no T cell epitopes in BALB/c or C57BL/6 mice in these regions (see Table S1 in the supplemental material). This was despite the presence of documented T cell epitopes in blocks 1 and 3 in humans (29, 43) as well as in both allelic forms of block 16 (14). However, this probably simply reflects the lesser capacity of inbred mice strains to recognize multiple peptide epitopes than outbred humans with a more diverse repertoire of antigen-presenting HLA molecules.

There was a sigmoidal relationship between the ELISA titer to MSP-1_{42} and the GIA of purified IgG. This suggests that the functional activity of the sera was related to responses to the 42-kDa C terminus. Using a reversal-of-inhibition assay, we have found the growth-inhibiting activity of the sera to be related to the presence of antibodies to the 19-kDa C terminus of MSP-1 (see Fig. S6 in the supplemental material). It has been shown previously that antibodies against all subunits of MSP-1 are capable of preventing parasite growth *in vitro* (58). Despite induction of antibodies to the conserved regions of MSP-1 by these virus-vectored vaccines, it was only the antibodies to MSP-1_{19} that appeared to be essential for growth-inhibitory activity in this study. Although PfM115 includes only the 3D7 allele of MSP-1_{19}, the antibodies induced by these vaccines demonstrated activity against the alternative allelic

FVO strain of *P. falciparum* as measured by ELISA and GIA assays. The inclusion of the second allele of MSP-1_{19} in PfM128 in mice led to stronger antibody induction in mice against the QKNG allelic variant and may therefore be of benefit for human vaccination.

Previous vaccines based on MSP-1 have utilized a panel of monoclonal antibodies in order to confirm vaccine antigen conformation and identity (41, 50). However, only one previous attempt has been made to alter the structure of the antigen to minimize the induction of blocking antibodies (16). We found that these amino acid changes abolished binding of one blocking monoclonal antibody (MAb 2.2) without abolishing inhibitory MAb binding or GIA activity. The abolishment of binding to blocking MAb 2.2 with the modified protein produced by the virus-vectored vaccines may or may not have been associated with a reduction in the induction of blocking antibodies in general following *in vivo* immunization. Further work is necessary to determine whether such changes will have a significantly beneficial effect in the development of effective vaccines based on MSP-1, given that such blocking antibodies appear to interfere with protection against malaria in humans (19). In C57BL/6 mice a $CD8^+$ T cell epitope was also detected within MSP-1_{19}, which may indicate that the removal of the disulfide bond aided antigen processing and presentation. However, we did not develop viral vaccines expressing the antigen PfM115 without the amino acid alterations and were thus unable to compare directly the response of this vaccine to that of a wild-type antigen construct. We also did not develop a viral vaccine based on the MSP-1_{42} region alone in order to determine whether inclusion of the conserved blocks was of benefit to overall immunogenicity. Testing of this construct in outbred populations with a greater repertoire of major histocompatibility complex (MHC) molecules will be of benefit in addressing this question.

Overall, the vectors and immunization approach described here should now provide an opportunity to assess the protective efficacy of strong T cell responses combined with high-level antibody responses against MSP-1 in humans. This could be assessed by blood-stage challenge (51) or by sporozoite challenge using infectious mosquito bites (56). The former allows lower-dose parasite challenges to be employed, but the latter may allow an added protective effect of T cells against MSP-1 expressed during merozoite development in the liver stage of the life cycle to be measured. The approach described here represents the beginning of a new *P. falciparum* blood-stage malaria vaccine strategy and also encourages the clinical assessment of both simian adenovirus-MVA and heterologous simian adenoviral vector regimens for other, diverse disease applications.

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A.L.G., S.C.G., A.V.S.H., and S.J.D. are named inventors on patent applications covering vectored malaria vaccines and immunization regimens. Authors from Okairòs are employees of and/or shareholders in Okairòs, which is developing vectored malaria vaccines. J.M.W. is a consultant to ReGenX Holdings and is a founder of, holds equity in, and receives a grant from affiliates of ReGenX Holdings; in addition, he is an inventor on patents licensed to various biopharmaceutical companies, including affiliates of ReGenX Holdings.

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